

Original Article

Median nerve electrical stimulation causes wake-promotion by downregulating the expression of γ -aminobutyric acid b receptor via orexins pathway in comatose rats caused by traumatic brain injury

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Received November 23, 2016; Accepted September 20, 2017; Epub October 15, 2017; Published October 30, 2017

Abstract: Our previous studies have shown that median nerve electrical stimulation (MNS) causes wake-promotion by activating the orexins system in traumatic brain injury (TBI) induced comatose rats. To better understand the mechanism of MNS induced wake promotion, the purpose of this experiment was to investigate the expression of γ -aminobutyric acid b receptor (GABA_bR) in the prefrontal cortex after MNS in comatose rats, as well as the relationship between GABA_bR and the orexins system. One hundred and twenty Sprague Dawley rats were divided into four groups: control group, TBI group, stimulated group and an antagonist group. We established TBI models by free fall drop. In the stimulated group, the TBI induced comatose rats were stimulated by the median nerve. In the antagonist group, comatose rats were given SB334867, an orexin receptor 1 antagonist, by intracerebroventricular injection and MNS. Then, the behavior changes were evaluated and the expression of GABA_bR was detected by the western-blot and immunohistochemistry at 6 h, 12 h and 24 h. Results showed that 5 of 30 rats were awakened in the TBI group, 22 of 30 awakened in the stimulated group and 13 of 30 rats were awakened in the antagonist group. The expression of GABA_bR in the prefrontal cortex in different groups was as follows: control group < stimulated group < antagonist group < TBI group ($P < 0.05$). Taken together, these data indicated that MNS has wake-promoting effects in comatose rats caused by TBI. One possible mechanism is that MNS might decrease the expression of GABA_bR via the orexins pathway.

Keywords: GABA_b receptor, orexins, median nerve electrical stimulation, coma, traumatic brain injury

Introduction

Traumatic brain injury (TBI) occurs in over 55 million people per year throughout the world and is a leading cause of death and disability [1]. Although the treatment of TBI has improved, there remain significant functional disturbances in subacute and chronic phases [2, 3]. A coma is a serious complication associated with TBI, for which there are no obvious clinical improvements for conventional treatments. Median nerve electrical stimulation (MNS) is a promising novel treatment for comatose patients with TBI [4-6]. However, the mechanisms of MNS induced wake-promotion are still poorly understood. The most current reports suggest that MNS causes wake-promotion by

affecting the secretion of neurotransmitters in the central nervous system (CNS) [7, 8].

CNS neurotransmitters such as orexins, including orexin-A and orexin-B, produced in the lateral hypothalamus (LHA) are associated with growth hormones, metabolic rate, autonomic function, food intake and sleep/wakefulness cycle [9]. They have two receptors, orexin receptor 1 and receptor 2 (OX1R and OX2R), which are important for wake-promotion [9]. On the contrary, γ -Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the CNS involved in sleep/wakefulness cycle [10, 11]. GABA is widely distributed in the CNS, specifically in the prefrontal cortex and hypothalamus [10]. It is reported that orexins could accelerate

Median nerve electrical stimulation causes wake-promotion

the switch from sleep to wakefulness by inhibiting the neurons of GABA [12, 13]. Furthermore, our previous studies have revealed that MNS could promote awakening by upregulating the expression of orexin-A and OX1R in the prefrontal cortex [14, 15]. Therefore, the purpose of this experiment was to investigate the relationship between MNS induced wake-promotion and the expression of GABA_BR as well as the function of the orexins pathway.

Material and methods

Overview of experimental animal group

One hundred and twenty male adult Sprague-Dawley rats (250-300 g) were commercially obtained from Animal Experimental Science Department of Nanchang University (Nanchang, Jiangxi Province, China) and kept in standard cages for a minimum of 1 week prior to any procedure. Food and water were continuously available. Animals were maintained at a constant temperature (23°C) in natural light. All experimental procedures conformed to the guidelines of the Animal Care and Use Committee at Nanchang University. Furthermore, all efforts were undertaken to minimize animal discomfort and reduce the total number of animals used. Following acclimatization, the animals were randomly assigned to four groups: control group (healthy rats), TBI group (comatose rats after TBI), stimulated group (comatose rats were given MNS) and an antagonist group (comatose rats were given MNS after intracerebroventricular injection with OX1R antagonist SB334867). Each group consisted of 30 rats.

Establishment of TBI induced coma model

We used the classic method, "Free fall drop", to establish TBI induced coma models [16]. The rats in TBI group, stimulated group and antagonist group were anesthetized with diethylether inhalation and allowed to breathe air spontaneously. After the anesthesia was introduced, rats were prone to a sponge pad and the skull was exposed via a 10 mm vertical incision in the middle of the head followed by disinfection. An iron spacer (diameter 10 mm, thickness 0.5 mm) was fixed on the skull between the coronal suture and the lambdoid suture by dental cement. The bottom of the iron spacer made an arc to coincide with the skull. When the rat

restored the sting reflex, a cylindrical impact hammer (diameter 10 mm, weighing 400 g) was dropped at a vertical height of 40-44 cm along with a vertical metal bar and hit the iron spacer fixed previously, causing a diffuse TBI model. Then, the sponge pad was immediately removed to avoid the second impact and the hit time was recorded. Animals were then disinfected and removed to cages after the incision was closed. An hour later, the degree of consciousness was assessed via a double-blind method according to the animals' sensory and motor functions as follows: I: Activities were free in cages; II: Activities decreased; III: Activities decreased with motor incoordination; IV: Righting reflex could be elicited but animals couldn't stand up; V: Righting reflex disappeared but animals could react to pain; VI: Animals had no reaction to any stimulation. We defined rats in degrees V and VI as a coma state [17]. These animals were brought into the assessment procedures. The sham operation and anesthesia were also measured in the control group.

Intracerebroventricular injection with OX1R antagonist SB334867

Intracerebroventricular injection of OX1R antagonist, SB334867, was conducted in the antagonist group. One hour before surgery, each rat received a pretreatment with gentamicin (0.1 mL/100 g, i. m.) and was anaesthetized with 10% chloral hydrate (0.3 mL/100 g, i. p.). Once anesthetized, the rats were prone to a stereotaxic frame (ZS-B/S, Beijing Zhongshidichuang development of science and technology Co., Ltd., China) and the skull was exposed via a 5-10 mm vertical incision in the middle of the head after disinfection. The bregma was exposed and marked with ink. The bregma was used as the zero point to look for the entry point of the needle. The lateral ventricle coordinates were: 1.0 mm posterior to the bregma, 1.5 mm lateral from the midline and 4.5 mm ventral to the skull surface, with the incisor bar set 3.2 mm below the interauricular line according to Paxinos & Watson rat brain atlas [18]. We marked coordinates with ink on the surface of the skull, then drilled the skull to the endocranium by dental drill under sterile conditions. The micro syringe was inserted vertically into the skull about 4.5 mm then rats received 5 µL SB334867 (Tocris Bioscience, Ellisville, MO, USA) 10 mg/kg dissolved in a 60:40 dimethyl

Median nerve electrical stimulation causes wake-promotion

Table 1. Effect of MNS on the recovery of consciousness in TBI-induced comatose rats

	Revived (n)	Coma (n)		(Mean \pm SD)
		Level IV	Level V	
Control group	30			1
TBI group	8	10	12	5.33 \pm 0.758
Stimulated group	22	5	3	3.80 \pm 1.064
Antagonist group	13	11	6	4.67 \pm 0.844

Control group: Healthy rats with sham operation and anesthesia. TBI group: Free fall drop was used to establish the model of TBI induced coma. Stimulated group: TBI induced comatose rats received stimulation with MNS. Antagonist group: Comatose rats received intracerebroventricular injection of OX1R antagonist SB334867 and MNS. Consciousness was classified into six levels and level V-VI defined as coma state. MNS: median nerve stimulation; TBI: traumatic brain injury.

sulfoxide (DMSO) solution over 1 min evenly. The needle was retained in the skull for 1 min before pulling out, after which the wound was sutured and disinfected. MNS was performed after the rats were restored from anesthesia. The same procedure in the antagonist group was conducted in a similar manner but without injection of SB334867 in the stimulated group.

Median nerve stimulation

Rats in the stimulated group and the antagonist group were treated with MNS by a low frequency electrical stimulator (ES-420, ITO Physiotherapy & Rehabilitation, Tokyo, Japan). Two acupuncture needles were inserted (depth 1 mm, angle 45°) in the middle of the right wrist joint with 5 mm distance and 15 mm distance after being sterilized, and connected with a stimulator. Ipsilateral thumb twitch was observed after the above procedure. The parameters of stimulation were as follows: Frequency, 30 Hz; pulse width, 0.5 ms; electric current, 1.0 mA; total stimulating time was 15 minutes [19]. Finally, these rats were returned to their cages with sufficient food and water before execution. Rats in the TBI group underwent the same surgical procedures as described above, but without any current output.

Western-blot

Five rats from each group were sacrificed with excess diethyl ether at 6 h, 12 h, 24 h after stimulation. The tissue samples of prefrontal cortex were homogenized by a Tissue Protein Extraction Kit (CW0891, Beijing Kangwei Biotechnology Co., Ltd., China). Kit contents

contained tissue protein extraction reagent 25 ml and protease inhibitors mixture 250 μ l. Homogenates were centrifuged at 12,000 \times g for 10 min at 4°C and supernatants were divided into small aliquots, stored at -80°C. The amount of total proteins in each sample was determined by the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA). Homogenate samples containing equal amount of proteins and loading buffer were boiled for 5 min in water and run on a 10% sodium dodecyl sulfate (SDS)/polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF membranes using a Mini trans-blot apparatus (Bio-Rad Laboratories,

Hercules, CA, USA) according to the manufacturer's instructions. Membranes were blocked for 4 h at room temperature with TBS-T buffer (150 mmol/L NaCl, 20 mmol/L Tris HCl, pH 7.4, 0.1% Tween-20) containing 5% milk. The blots were incubated with anti-GABA_BR antibody (ab131417, Abcam, HK Co., Ltd., China) diluted 1:500 in TBS-T containing 5% milk at 4°C for overnight. After the incubation, the membranes were washed three times with TBS-T and incubated for 1 h with horseradish peroxidase conjugated goat anti-rabbit IgG (H+L) (ZB-2301, Beijing Zhongshan-Golden Bridge Biotechnology Co., Ltd., China) diluted 1:3000 in TBS-T at room temperature. The concentration of proteins was detected by BCA. A relative level of GABA_BR protein was assessed by absorbance and Quantity One software (Bio-Rad, Hercules, CA, USA) to quantify the protein bands. The results were expressed as the mean \pm standard deviation of the ratio of immunoreactivity normalized by β -actin.

Immunohistochemistry

10% chloral hydrate (200 mg/kg, i.p.) was used to ensure the rats remained under deep anesthesia, then the rats were perfused with 4% paraformaldehyde. After that, the brains were removed and fixed in 4% paraformaldehyde for 8 h, then cut into 40 μ m coronal sections with a sliding microtome. After dewaxing, we placed sections in 3% hydrogen peroxide. Sections were dropped in antigen repairing liquid followed by normal goat serum blocking solution. Primary antibodies were incubated overnight followed by second biotinylated antibody and streptavidin-biotin complex (SABC). The dye

Median nerve electrical stimulation causes wake-promotion

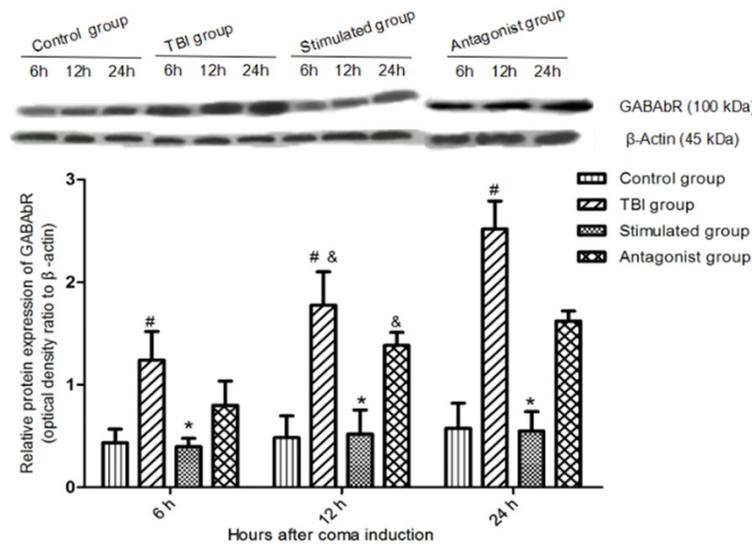


Figure 1. GABA_BR expression in the prefrontal cortex after median nerve electrical stimulation in comatose rats caused by traumatic brain injury (Western blot analysis). Relative protein levels of GABA_BR were assessed by densitometry. Data are expressed as the mean \pm SD in each group and one-way analysis of variance was performed (n=10). #P < 0.05, TBI group, compare with control group and/or stimulated group at 6 h, 12 h, 24 h. *P < 0.05, stimulated group, compare with antagonist group at 6 h, 12 h, 24 h. &P < 0.05, 6 h and/or 24 h, compared with 12 h in TBI group and antagonist group. GABA_BR, gamma aminobutyric acid b receptor; TBI, traumatic brain injury.

3-3' diaminobenzidine (DAB) and hematoxylin were used as final staining. After being mounted the pictures were observed by a Nikon Eclipse E 600 light microscope, and photographed by a Colpix 8400 digital camera. Original magnification \times 400. The evaluation standard was as follows: A was positive cell number classification (0-1%=0, 1-10%=1, 10-50%=2, 50-80%=3, 80-100%=4) and B was positive cell staining intensity classification (0, negative; 1, weakly positive; 2, positive; 3, strongly positive). The results were comprehensive score A*B [20].

Statistical analyses

Western blot data were expressed as the mean \pm SD, and immunohistochemistry data as the mean rank. One-way analyses of variance with Turkey's test were performed for comparison of consciousness in different groups as well as for comparison of results from western-blot. The Kruskal Wallis-Test was used for comparison of Immunohistochemistry results. All data were analyzed with SPSS 17.0 software (SPSS, Chicago, IL, USA). P < 0.05 was used to indicate statistical significance.

Results

Evaluation of the consciousness grade after MNS in TBI-induced comatose rats

Behavioral assessments were conducted among the four groups by double-blind reviewers one hour after the experiment was finished. A total of 11 rats were dead and excluded from this experiment. All rats in the control group were class I. After TBI brief apnea (about 5-10 s), exophthalmoses and beard tremor occurred among the rats in the TBI group. Part of the rats presented with tic of the limbs and urinary incontinence, followed by a comatose state. Results showed that only 5 rats experienced recovery from the coma in the TBI group (IV degree: 8, V degree: 10, VI degree: 12) and the average level was 5.33 ± 0.758 . Rats

in the stimulated and antagonist group emerged with reactions such as accelerated respiration, eyes opening, right limb tics, retraction and scrolling. In the stimulated group, 22 rats awakened (III degree: 17, IV degree: 5, V degree: 5, VI degree: 3) and the average level was 3.80 ± 1.064 . However, 13 rats awakened (III degree: 4, IV degree: 9, V degree: 11, VI degree: 6) in the antagonist group and the average level was 4.67 ± 0.844 . A comparison of the rat's consciousness among four groups was ordered as follows: control group < stimulated group < antagonist group < TBI group (P < 0.05) (Table 1).

Expression of GABA_BR by western-blot

Western blot data showed that the trends of GABA_BR expression in the prefrontal cortex among four groups at 6 h, 12 h, 24 h was as follows: stimulated group < control group < antagonist group < TBI group. However, there was statistical significance between the TBI group and the control group or stimulated group at different time points (P < 0.05). The stimulated group and the antagonist group were also significantly different at 6 h, 12 h, 24 h (P < 0.05).

Median nerve electrical stimulation causes wake-promotion

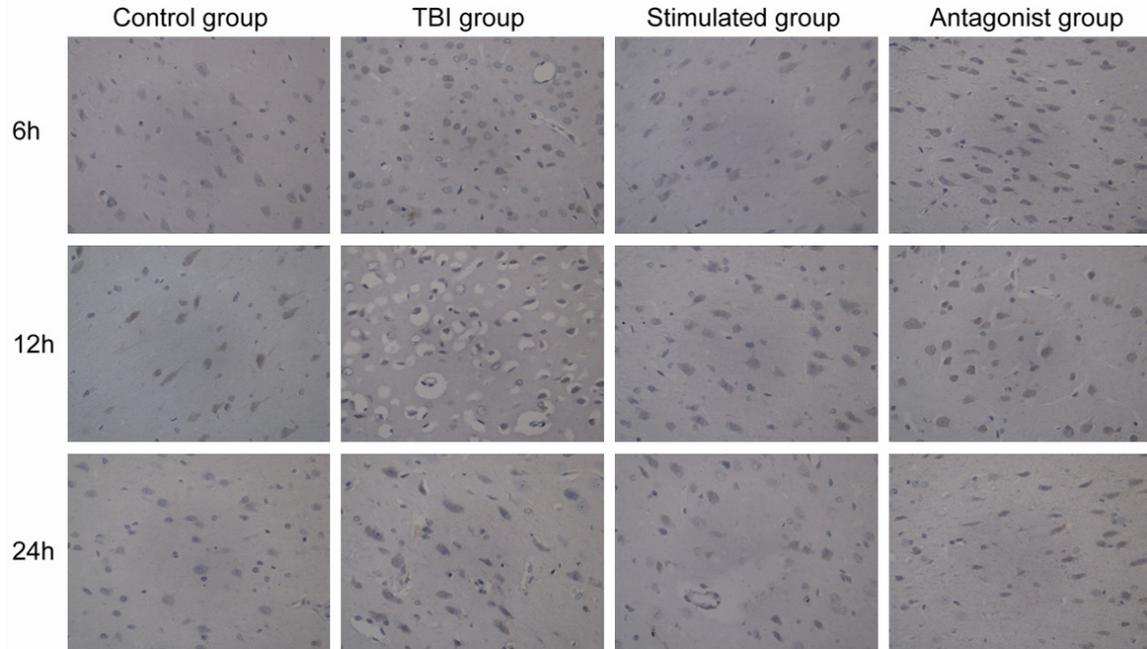


Figure 2. Representative photomicrographs GABA_BR immunoreactivity localization in the prefrontal cortex. Distribution of GABA_BR immunoreactivity in the control group, TBI group, stimulated group and antagonist group with 6 h, 12 h, 24 h (400 times magnification). GABA_BR immunopositive cells were stained and exhibited circular patterns. GABA_BR, gamma aminobutyric acid b receptor; TBI, traumatic brain injury.

Furthermore, a within-group comparison suggested that the expression of GABA_BR was as follows: 6 h < 12 h < 24 h in the TBI group ($P < 0.05$), antagonist group ($P < 0.05$), control group ($P > 0.05$) and stimulated group ($P > 0.05$) (Figure 1).

Expression of GABA_BR by immunohistochemistry

GABA_BR distributed in the cytoplasm of neurons in the prefrontal cortex and all photomicrographs of GABA_BR immunoreactivity were examined by image analysis. Significant differences were observed in GABA_BR immunoreactivity in four groups as follows: stimulated group (80.00) < control group (87.38) < antagonist group (120.26) < TBI group (129.88) ($\chi^2=98.798$; $P < 0.001$) (Figure 2). There was also a significant increase in GABA_BR expression at 6 h (90.85), 12 h (113.16), and 24 h (113.26) ($\chi^2=96.825$; $P < 0.001$) (Figure 2).

Discussion

The main objective of this study was to identify the connection between MNS and GABA_BR expression as well as the role of the orexins pathway. Results from this experiment demon-

strate that 1) MNS could promote improved recovery of consciousness in comatose rats caused by TBI; 2) MNS downregulates the expression of GABA_BR in the prefrontal cortex; 3) MNS decreases the GABA_BR expression via the orexins pathway (Figure 3).

A coma is a serious disorder of consciousness caused by various mechanisms such as TBI, cerebrovascular accident and alcoholism [21]. At present, we believe that the pathogenesis of a coma could be resultant from damage to the Ascending Reticular Activating System (ARAS) in the brainstem and neurotransmitter imbalances between wakefulness/sleep [14, 15, 19]. Clinical treatment methods for comas include drugs, hyperbaric oxygen, cell transplantation, music therapy, sensory stimulation and MNS [7, 22, 23]. It was reported that MNS could significantly promote the recovery of comatose patients caused by TBI during the early phase [23]. We believe that MNS could accelerate the recovery from a coma by influencing the secretion of related neurotransmitters [8, 23].

GABA is the most important inhibitory neurotransmitter and is widely distributed throughout the CNS. Neurophysiologic studies suggest

Median nerve electrical stimulation causes wake-promotion

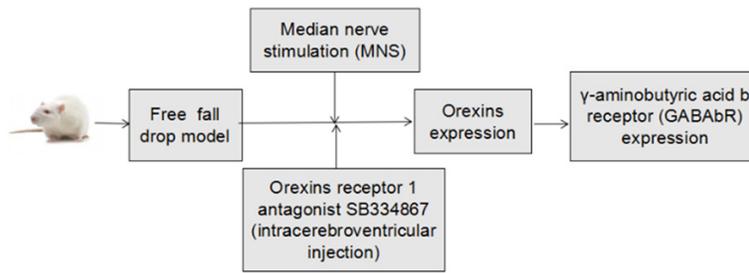


Figure 3. Schematic overview of the mechanisms for median nerve electrical stimulation induced wake promotion in traumatic brain injury induced comatose rats.

that GABAergic neurons in different brain regions work together to promote sleep [24]. GABA has two important receptors that may play a role in sleep promotion, one of which is the G protein coupled GABA_b receptor (GABA_bR) [25, 26]. Previous studies have shown that GABA_bR expression in the neocortex and thalamus significantly increased in rats with drowsiness [27, 28]. Furthermore, GABA agonist baclofen could promote sleep and reduce cataplexy by activating GABA_bR in murine narcolepsy [29]. On the contrary, losses of GABA_bR help mice delay hypersomnia and inhibit EEG slow (delta) waves during non-rapid eye movement sleep (NREMS). Finally, decreases in GABA_bR promote theta waves during awakening, which are important for retaining learned information [30]. These data are partially consistent with our present finding, which showed that GABA_bR expression and recovery from the coma were both higher in the TBI group than the control group. Therefore, the above observations indicate that GABA_bR plays an important role in sleep-promotion.

In the present study, we behaviorally evaluated consciousness after the TBI-induced coma model. The numbers of rats recovering from comas were higher after MNS than those with no intervention, consistent with our previous studies [14, 15, 19]. Furthermore, expression of GABA_bR was also decreased in the stimulated group compared with the TBI group at different points. These data suggest that MNS might decrease the expression of GABA_bR to promote wakefulness. However, the level of GABA_bR after MNS increased gradually with time at 6 h, 12 h, 24 h, which indicates that the effects of MNS for comatose rats is decreased with time. These data revealed that downregulation of GABA_bR expression might play an

essential role in MNS induced wake-promotion.

Orexins (Orexin-A and Orexin-B) are synthesized in the lateral hypothalamus (LHA) and project widely to many brain areas such as the prefrontal cortex, basal ganglia and ventrolateral preoptic nucleus (VLPO) [31]. It is reported that orexergic neurons of LHA could inhibit GABAergic neurons of VLPO to promote awakening

from sleep [32, 33]. Anatomical connections between orexergic and GABAergic neurons have also been demonstrated [34]. Furthermore, our previous studies suggest that orexins play a key role in wake-promotion of MNS [14, 15, 19]. In this experiment we have demonstrated that comatose rats with OX1R antagonist, SB334867, had significantly higher GABA_bR expression levels in the prefrontal cortex. These results corroborated the effects of the consciousness evaluations, which indicated that the numbers of rats re-awakened from coma in the antagonist group were less than in the stimulated group. We conclude that the orexins pathway plays an essential role in promoting wakefulness via the expression of GABA_bR following MNS.

Previous studies have shown that GABA_bR expression may be different depending on the time of day [26, 35]. Our experiment indicates that GABA_bR expression was significantly increased at 6 h, 12 h, 24 h in the TBI and antagonist groups with an increased trend in the control and stimulated groups, though not statistically significant. We believe this might represent the physiological secretion cycle of GABA because the timing of rat executions was not unified, depending on their individual time (6 h, 12 h, 24 h) following TBI or MNS.

There are several limitations to this study. We recognize that other methods of measuring the consciousness grade which may be more accurate, such as evoked potential and electroencephalogram, could have been employed. In addition, further exploration is needed to determine whether different stimulation parameters and therapy periods may have an effect on comatose rats after TBI. Finally, the relationship between MNS and other neurotransmit-

ters such as glutamate, norepinephrine and dopamine also should be studied in the future.

In conclusion, we investigated the expression of GABA_BR in the prefrontal cortex after MNS in comatose rats caused by TBI, also illuminated the functional connection between orexins and GABA_BR by microinjecting OX1R antagonist (SB334867) into the cerebral ventricle. Our study reveals, for the first time, that MNS induced wake-promotion is due, at least in part, to the downregulation of GABA_BR expression via the orexins pathway, and provides strong evidence for MNS promotion of wakefulness in the treatment of comatose patients with TBI.

Acknowledgements

This study was funded by the National Natural Science Foundation of China (No. 81260295), and the Natural Science Foundation of Jiangxi Province of China (No. 20132BAB205063).

Disclosure of conflict of interest

None.

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Median nerve electrical stimulation causes wake-promotion

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